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## Multiple Sequence-Specific Transcription Factors Modulate Cytomegalovirus Enhancer Activity In Vitro

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**The possibility of DNA-binding proteins interacting in vitro with the polymerase II transcriptional machinery was explored by using a competition assay with individual target sequences for enhancer-binding factors. Transcription factors binding to at least five specific enhancer sequences mediate the activity of the human cytomegalovirus immediate-early 1 gene in vitro. Furthermore, our data suggest that individual DNA-bound enhancer factors can interact with the promoter transcription complex.**

The human cytomegalovirus (CMV) immediate-early 1 gene (IE1) enhancer is a strong potentiator of transcription both in vivo and in vitro (1, 2, 10). The sequence structure of the enhancer region, which extends from about nucleotide –65 to –530, exhibits a highly modular complexity. We have reported previously that five distinct sequence motifs within the enhancer region are targets for specific nuclear proteins (2). Here we present an approach for studying the involvement of these sequence-specific DNA-binding proteins with the transcriptional machinery. This involves the synthesis of oligonucleotides corresponding to specific protein-binding sites and a study of their effect in the in vitro transcription competition assay (4, 8, 9, 12). An advantage of this approach is that it can be used to directly monitor the transcriptional machinery in a crude in vitro system that upon further fractionation might lose transcription factors, which may go undetected. Also, the assay avoids an alteration of the DNA sequence structure that may be important for enhancer activity. The assay also minimizes the possibility of competition for several proteins capable of interacting with larger restriction fragments (2, 8, 9, 12).

In pursuit of this approach, oligonucleotides corresponding to the target sequences (Fig. 1A) were synthesized and prepared for the in vitro transcription competition assay. The in vitro transcription assay and the preparation of HeLa cell nuclear extract have been described previously (2). The templates, pCMV(–524)CAT and pCMV(–65)CAT, used for runoff analysis were a complete *PvuII*-*HindIII* restriction enzyme digest (2). The amount of template present in each in vitro synthesis was 300 ng per 25- $\mu$ l reaction. The nuclear extract (6  $\mu$ l), in 21  $\mu$ l of buffer containing 12 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.9), 10% (vol/vol) glycerol, 1.6 mM dithiothreitol, 0.12 mM EDTA, 60 mM KCl, 2 mM MgCl<sub>2</sub>, and 5 U of placental ribonuclease inhibitor, was initially incubated for 10 min at 4°C with or without the appropriate competitor DNA, as indicated. The template and nucleoside triphosphates were subsequently added, and the in vitro synthesis was allowed to proceed for 30 min at 30°C. The synthesized RNA was processed and analyzed on a 4% sequencing gel as described previously (2).

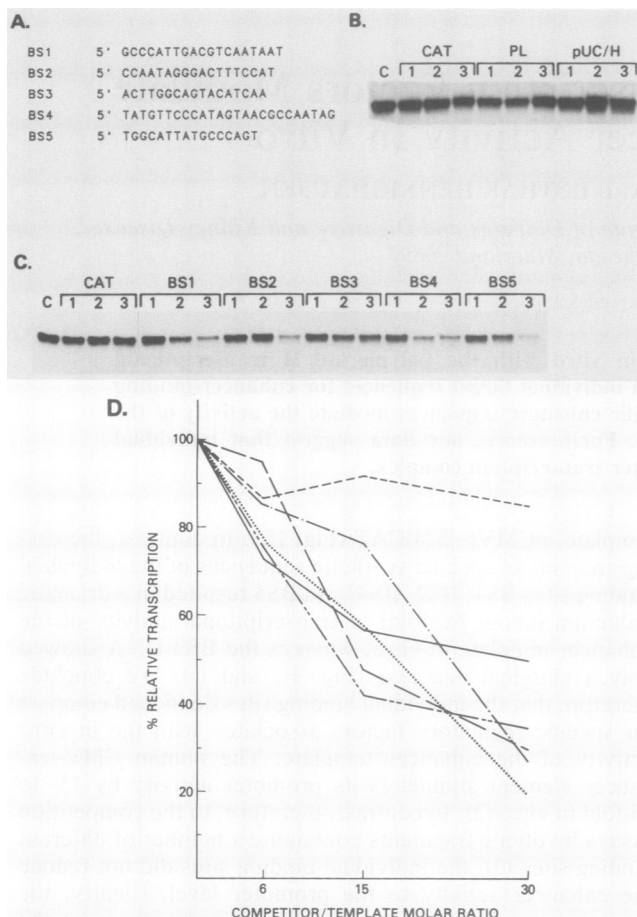
Increasing the amount of several different nonspecific DNAs in the transcription reactions did not significantly alter the transcriptional activity of the enhancer-containing

template, pCMV(–524)CAT (Fig. 1B). In contrast, increasing amounts of specific synthetic sequences of the individual binding sites BS1, BS2, BS4, and BS5 resulted in a dramatic reduction (about fivefold) of transcriptional activity of the enhancer template in vitro, whereas the BS3 DNA showed only a marginal decrease (Fig. 1C and D). We conclude therefore that the individual binding sites do indeed compete for specific regulatory factors associated with the in vitro activity of the enhancer template. The human CMV enhancer element stimulates its promoter activity by 15- to 25-fold in vitro (2). In contrast, therefore, to the competition assays involving fragments containing a number of different binding sites (2), the individual binding sites did not reduce the enhancer activity to the promoter level. Clearly, the involvement of multiple transcription factors increases the combinatorial possibilities of modulating the activity of the enhancer element. Recognition motifs similar to the sequences of BS1 and BS2 have been functionally characterized in other enhancer elements (5, 6). Moreover, we demonstrate the importance of three binding sites, BS3, BS4, and BS5, as novel recognition sequences for specific transcription factors.

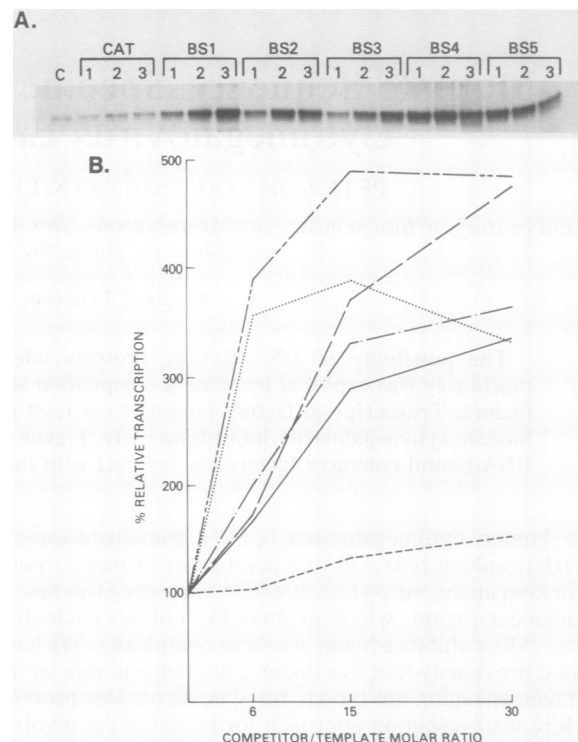
To examine the effect of the enhancer-protein target sequences with the CMV promoter element, competition assays were carried out with the plasmid pCMV(–65)CAT, which lacks the enhancer. Under the same conditions as used with the enhancer template, the addition of nonspecific DNA did not reduce but increased slightly the level of specific transcription (Fig. 2). In the presence of DNA containing the specific binding sites, a greater increase of specific transcription over the level with the nonspecific competitor was observed (Fig. 2A and B). We did not observe this effect in competition assays with the adenovirus major late promoter. With increasing amounts of BS2 and BS3 DNA, the level of specific transcription remained constant, whereas BS1, BS4, and BS5 increased slightly the level of transcription to the same extent as nonspecific DNA (Fig. 3). Additionally, the level of transcription from the murine whey acidic protein gene promoter was not altered with nonspecific, BS2 and BS3 DNAs but was reduced significantly with BS4 and BS5 and was slightly increased with BS1 (unpublished observations).

The observation that specific-enhancer-sequence-binding transcription factors can increase CMV promoter activity is subject to several interpretations. At this point, we cannot exclude partial, nonspecific effects of such binding sites on

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**FIG. 1.** (A) The sequences of one strand of the synthetic binding sites are shown. BS1 corresponds to the 19-base-pair (bp) repeat element (–469 to –451); BS2 corresponds to the 18-bp repeat element (–428 to –411); BS3 corresponds to the 17-bp repeat element (–373 to –357) as previously described (1, 2); BS4 (–446 to –422) corresponds to site III (2), and BS5 (–299 to –284) corresponds to site I (2). The nucleotide positions in the parentheses above refer to the location of the sequence in the human CMV IE1 enhancer with respect to the transcriptional start site. The synthetic single-stranded oligonucleotides were treated with kinase, annealed to the complementary oligonucleotide, and cloned into the polylinker of pUC18 or pUC19. BS1 and BS2 oligonucleotides were from B. Fleckenstein. Fragments containing the individual binding sites were gel purified from their respective plasmids after cleaving with *EcoRI* and *HindIII*. (B) Effect of nonspecific DNA fragments on the transcriptional activity of pCMV(–524)CAT (2) in an in vitro transcription competition assay. Lane C, transcription of pCMV(–524)CAT template in the absence of competitor DNA; lanes 1 through 3, effect of incubating the nuclear extract for 10 min with increasing molar amounts (lane 1, 6 to 8; lane 2, 12 to 15; and lane 3, 25 to 30) of nonspecific competitor DNA before adding the template: CAT, a 250-bp *XbaI*-*NcoI* fragment (spanning chloramphenicol acetyltransferase gene sequences) from pCMV(–524)CAT; PL, the *EcoRI*-*HindIII* polylinker fragment from pUC19; and pUC/H, pUC19 DNA cut with *HaeIII*. The amount of competitor DNA is expressed as a molar ratio with respect to the template. (C) Effect of specific DNA-binding sites on the transcriptional activity of pCMV(–524)CAT in vitro. Lane C, pCMV(–524)CAT in the absence of competitor DNA; lanes 1 through 3, nuclear extract incubated for 10 min with increasing molar amounts of various DNA (molar ratio as in lanes 1 to 3 of Fig. 1B) before the template was added. The competitor DNA used is indicated above the numbered lanes. (D) Effect of the different competitor DNAs on in vitro transcription of pCMV(–524)CAT, summarizing the results from



**FIG. 2.** (A) Effect of nonspecific and specific sites of protein-DNA interaction on the transcriptional activity of pCMV(–65)CAT in vitro. The lanes are as described in the legend to Fig. 1C. (B) Quantitative effects of the different competitor DNAs on in vitro transcription of pCMV(–65)CAT. The lines are defined in the legend to Fig. 1D.

the transcriptional machinery associated with the promoter. Since a defined regulatory DNA sequence can be recognized by more than one protein, it is also possible that the binding sites used in this experiment are able to titrate the activity of a repressor function in connection with the promoter complex. Alternatively, these binding sites could *trans*-stimulate the transcription complex associated with the promoter. Indeed, the potential for *trans*-stimulation is in agreement with the looping model which mechanistically necessitates the cooperative interactions between proteins bound to DNA (3, 7). Interestingly, a similar increase in specific transcription has been observed with a simian virus 40 early promoter template missing its enhancer sequences in the presence of certain specific enhancer fragments (12). Further studies are in progress to distinguish between the possible mechanisms involved.

several experiments similar to those shown in Fig. 1C. For each experiment, quantitation was achieved by densitometric scanning of the autoradiograms. The relative transcriptional activity of a template is plotted as a function of increasing competitor/template molar ratio and expressed as a percentage of its transcriptional level in the absence of competition. The lines correspond to competition with the following DNAs: CAT (– – – – –), BS1 (— — —), BS2 (— · — · —), BS3 (— — —), BS4 (— — —), and BS5 (· · · · ·). The structures of the recombinants used as templates have been described previously (2).

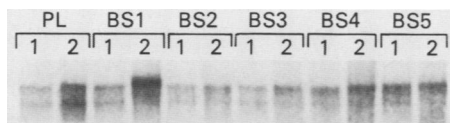


FIG. 3. Effect of the various competitor DNAs on the level of specific transcription from the adenovirus major late promoter. The plasmid pSmaF (11) was linearized with *Hind*III and the *in vitro* transcription competition assay was performed as described in the legend to Fig. 1, except that 8 mM MgCl<sub>2</sub> was used. Lanes 1 and 2, *In vitro* competition assays performed in the presence of 12 to 15 and 25 to 30 molar amounts, respectively, of the different DNAs. The DNA lanes are defined as described in the legend to Fig. 1A.

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